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Bioprospecting for protease-producing bacteria in mangrove ecosystems: Screening, isolation, and application studies

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ABSTRACT

Proteases constitute an important group of enzymes widely utilized in industrial processes due to their versatility for various applications. These applications range from their use in textiles and detergents to food processing and pharmaceuticals. Recently, bacterial proteases have garnered considerable attention for commercial applications. This interest stems from their ease of cultivation, simplicity in genetic modification, and low production costs, making them highly useful for commercial purposes. The unique conditions of mangrove soils harbor microorganisms that can produce enzymes and metabolites with valuable industrial properties that are less explored. In this study, bacterial strains were isolated from soil samples taken from the Pichavaram mangrove forest, Tamil Nadu, India, and screened for their ability to produce protease. Among those tested, one isolate (C2) showed the highest protease production after 96 h of incubation, achieving 633.14 U/mL crude enzyme (total enzyme activity). The isolate C2 was further characterized using 16S *rRNA* gene sequencing, which confirmed it as *Fictibacillus phosphorivorans*. In addition to measuring protease activity, this work assessed the effectiveness of crude enzyme in removing blood stains and its ability to break down keratin, which suggests potential uses in feather degradation and waste management. Mangrove ecosystems harbor highly diverse microorganisms adapted to extreme and fluctuating conditions, such as salinity, tidal changes, and oxygen limitation. The high organic matter content in mangrove sediments promotes the microbial production of extracellular proteases, facilitating nutrient cycling. These environmental pressures drive the evolution of proteases with enhanced stability and unique catalytic properties, making mangroves promising hotspots for novel protease-producing microbes. Overall, this research emphasizes the potential of bacteria found in mangrove environments as sources of important industrial enzymes. It also highlights the need to explore different ecological niches to find new microbial resources for biotechnological advancements.

1. INTRODUCTION

Proteases are a class of enzymes that catalyze the hydrolysis of peptide bonds in proteins. These enzymes are sometimes called as peptidases or proteinases; they are pivotal in many commercial and physiological activities. According to their catalytic functions, proteases are divided into four main categories: Metalloproteases, aspartic, cysteine, and serine proteases. Their unique properties and substrate specificity allow them to be utilized in a variety of sectors that include food, medicines, detergents, and leather processing [1]. Being one of the three largest classes of industrial enzymes, microbial proteases have historically dominated the industrial enzyme market, accounting for over 20% of global enzyme sales and being valued at $2.767 million in the pharmaceutical sector by 2019 [2]. Proteases of microbial origin are readily available on the market due to their high yield, low time and space requirements, ease of genetic manipulation, and affordability, making them well-suited for biotechnological applications [3].

Mangroves are a type of tropical coastal biome found in areas where land meets the sea [4]. They are found in river deltas, tropical and subtropical intertidal estuaries, and offer extremely delicate and dynamic ecosystems [5]. This ecosystem is a unique and significant coastal wetland because it forms a complex and wide-ranging food web, providing food, breeding grounds, and shelter for fish, birds, zoobenthos, and plankton [6]. They serve as a barrier against wind, waves, water currents, and numerous other harsh weather phenomena, such as soil erosion and ocean swell, and they cover around 25% of the world's tropical coastline [7]. Globally, mangroves make up between 60% and 70% of tropical and subtropical coastlines. They are unique intertidal ecosystems. They are valued for their remarkable ecological significance and productivity, providing a diverse range of aquatic and terrestrial life forms, as well as a wide variety of plant and animal species. These ecosystems' high concentrations of nutrients and organic carbon provide ideal circumstances for microorganisms that are suited to varied environmental conditions and mild salinity [8]. The significant nutritional transformations in mangrove ecosystems are caused by the

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activity of several microbial communities, which are supported by a richness in carbon and other nutrients. By utilizing both aerobic and anaerobic processes, these microbes break down organic waste to produce protein-rich detritus that other organisms can consume. These bacterial intricate relationships preserve the mangroves' ecological balance and nutritional health [5]. Microorganisms are indispensable in the mangrove ecosystem, as they sustain the environment and have been exploited for their applications in producing valuable biotechnological products. Investigating the microbial communities in this ecosystem is crucial for elucidating their ecological function and paves the way for the isolation and characterization of unidentified protease-producing bacterial strains with promising applications [9].

In this present study, bacteria were isolated from mangrove soil, Pichavaram, Tamil Nadu, and were screened for protease production with application studies involving blood stain removal and chicken feather degradation.

2. MATERIALS AND METHODS

2.1. Sample Collection

Soil samples were collected from a depth of 4 cm at the mangrove forest, Pichavaram, Tamil Nadu, under sterile conditions to prevent contamination, and were brought to the lab in a polyethene bag stored at 4°C in a cold refrigerator for future use [10].

2.2. Isolation of Bacteria

One gram of soil was taken and dissolved in 10 mL of water, and then serially diluted up to 10^{-5}. From this, 100 µL of the sample was inoculated into nutrient agar plates in a sterile laminar hood and incubated at 37°C for 24 h inside the incubator [11].

2.3. Screening of Bacteria Producing the Protease Enzyme

The proteolytic potential of bacterial strains was investigated on skim milk (SM) agar plates with two following compositions, one with 2.8% (w/v) SM powder, 0.5% (w/v) tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) glucose, and 2% (w/v) agar, and the other with a modified SM powder composition at 1% (w/v) (other components remains the same). The media pH was adjusted to 7, and two morphologically dominant colonies were streaked as single lines on the two segments of the petri plate and incubated at 37°C for 4 days.

2.4. Quantitative Enzyme Assay for Protease

One mL of overnight culture was used to inoculate 100 mL of S.M broth with the following composition (0.5 g of peptone, 1 g of sodium chloride [NaCl], 1 g of skimmed milk powder, 1 g of yeast extract) in 100 mL of distilled water, with pH adjusted to 7.2. Sigma Aldrich method was employed to quantify the protease activity using casein as the substrate, 1 mL of crude enzyme was mixed with 1 mL of 1% casein incubated at 37°C for 30 min, the reaction was terminated upon addition of 10% trichloroacetic acid, the mixture was allowed to set at room temperature for 10 min and centrifuged at 10,000 rpm for 10 min, from the supernatant 1 mL was taken and to that 5 mL of sodium carbonate solution and 1 mL of Folin's reagent was added and incubated at 37°C for 20 min, absorbance were read at 660 nm, the calculation of enzyme activity was done using tyrosine as standard [12]. One unit (1U) of enzyme activity refers to the amount of enzyme that converts 1 µmol of substrate into product each minute under specific assay conditions, including pH, temperature, and substrate concentration [13].

Total enzyme activity (U/mL) = µMoles of tyrosine equivalent release × Total volume of assay (mL)/Total volume of enzyme used (mL) × Time of assay × Volume in cuvette (mL).

2.5. Molecular Characterization

DNA from the proteolytic bacteria was isolated using GeneiPure™ bacterial DNA purification kit as per the manufacturer's protocol (Genei™, Bengaluru, India). Using the 16S rRNA-F and 16S rRNA-R primers (Universal 16S rRNA bacterial primers 27F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1392R [5'-GGTTACCTTGTTACGACTT-3']), a portion of the 16S *rRNA* gene was amplified, resulting in a single, unique 1500 bp polymerase chain reaction amplicon that could be resolved on an agarose gel. To remove impurities, the amplicon was cleaned. The BDT v3.1 Cycle Sequencing Kit was used on an ABI 3730xl Genetic Analyzer to conduct sequencing experiments in both directions using the same primers. Using alignment tools, forward and reverse reads were combined to create a consensus sequence. Basic Local Alignment Search Tool (BLAST) was used to compare. The sequence was submitted to the National Center for Biotechnology Information (NCBI) GenBank database, and an accession number was obtained. Clustal W was used to align the top ten matching sequences. MEGA 11 produced a phylogenetic tree and a distance matrix.

2.6. Washing Test for Detergent Properties

Protease enzymes are known to be effective in removing stains from clothes. To assess their efficiency and compatibility with detergents in blood stain removal, white fabrics were cut into 4 cm × 4 cm swatches and stained with 0.5 mL of chicken blood (collected from local slaughter houses with 2 mg of ethylenediaminetetraacetic acid per mL of blood as an anticoagulant). A variety of configurations were tested in four different setups. Tap water (control), local detergent + distilled water, crude enzyme + detergent + distilled water, and crude enzyme + distilled water. Cotton swatches were immersed in the corresponding solutions for 30 min at 40°C, and the cloths were visually observed for stain removal [14].

2.7. Screening for Keratinolytic Properties by Feather Degradation

Keratinolytic activity was evaluated by cultivating bacteria in minimal media supplemented exclusively with chicken feathers, since it contains keratin proteins. The composition of the minimal media was optimized based on the formulation by Stanly *et al*. The chicken feathers used in this study were collected from a local slaughterhouse, S.G. Palya, Bengaluru. Chicken feathers were collected, washed multiple times with distilled water, dried at 60°C and stored for future use. The media components included potassium dihydrogen phosphate (K_2HPO_4): 0.03 g, KH_2PO_4: 0.04 g, NaCl: 0.05 g, and 1 g of clean dried feathers dissolved in 100 mL of distilled water. Minimal media supplemented with chicken feathers was autoclaved at 121°C for 15 min. Then, 10 mL of overnight culture (1×10^8 colony-forming unit/mL) was added to the media under aseptic conditions inside a laminar hood, and the mixture was incubated at 37°C with shaking (125 rpm) for 4 days [15]. To estimate the percentage of keratinolytic activity, the chicken feather hydrolysate was centrifuged at 10,000 rpm for 10 min, and undegraded feathers were washed thoroughly with distilled water, dried at 60°C (until constant weight), and weighed.

Keratinolytic percentage is determined by the formula:

Feather degradation (%) = (Weight of the feather before degradation–Weight of the feather after degradation/Weight of the feather before degradation) × 100 [16].

3. RESULTS AND DISCUSSION

3.1. Bacterial Isolation

Microbial colonies from the soil collected from the mangrove forest, Pichavaram, Tamil Nadu, were isolated using the spread plate method under sterile conditions; Four distinct colonies were obtained; two morphologically dominant isolates (C1 and C2) were selected for primary protease screening.

3.2. Primary Screening for Protease-Producing Bacteria

In SM agar media supplemented with 2.8% SM powder, only one isolate (C2) produced a zone of clearance, whereas in the other media supplemented with 1% SM powder, both isolates (C1 and C2) produced a zone of clearance, as shown in Figure 1 the isolate C2 that exhibited maximum protease activity (as visually evident from the zone of clearance) in 2.8% SM powder was subjected to further studies.

3.3. Protease Enzyme Quantification Assay

Isolate C2 showing high zone of clearance in 2.8% SM media was selected for further protease quantification assay, following the centrifugation at 10,000 rpm for 15 min the supernatant obtained is used as crude enzyme and their total enzyme activity has been assessed from 24 h to 120 h, during this prolonged period the maximum protease activity was obtained at 96 h with 633.14 units/mL, after 96 h the activity of enzyme was observed to be decreasing [Figure 2].

Similarly, in a study by Sharma *et al*., the *Bacillus aryabhattai* strain K3 exhibited extremely high protease activity, reaching 622.64 U/mL when lactose was provided at a concentration of 10 g/L as the sole carbon source. They studied the effect of various sugars on enzyme production and concluded that among the carbon sources tried, lactose was far superior to glucose, galactose, and starch in inducing the production of protease. This is indicative of the view that lactose may act as an efficient inducer of protease synthesis in *B. aryabhattai* K3, either by relieving catabolite repression or by providing a better metabolic flux toward the biosynthesis of the enzyme [17].

The results of the study conducted by Sun *et al*., clearly indicate that the nutrient composition and the availability of metal ions are important factors affecting alkaline protease production by *Bacillus subtilis*. Among various carbon sources tested, corn starch supported maximum enzyme production, with peak alkaline protease activity of 768 U/mL during the stationary phase. The result was significantly different from that obtained using cornmeal ($P = 0.02$) and maltodextrin ($P = 0.03$), indicating that cornstarch provides a better carbohydrate profile for *B. subtilis* metabolic pathways associated with protease biosynthesis. The enhancement probably results from its gradual hydrolysis and steady release of glucose, maintaining enzyme production during the later phases of growth [18].

In another study by Feyissa, *Bacillus pumilus* DT-15 exhibited a distinct pattern of time-dependent protease secretion, achieving its maximum protease activity of 506 ± 0.037 U/mL at 60 h of incubation. The lowest yield of the enzyme (62 ± 0.014 U/mL) was obtained at 12 h, indicating that early growth phases are unsuitable for maximum protease release [19]. The enhanced protease and keratinase activities observed in the isolates can be directly linked to the unique environmental conditions of mangrove soils, characterized by high organic matter content, fluctuating salinity, periodic tidal inundation, and variable oxygen availability. These stresses exert strong selective pressure on resident microorganisms, favoring the production of extracellular enzymes that are stable and efficient under extreme conditions. Consequently, mangrove-derived microbes have evolved robust proteolytic systems to facilitate nutrient acquisition and survival, explaining the high enzyme activities recorded in this study [20].

For industrial uses, the protease and keratinase from *Fictibacillus phosphorivorans* can be further refined through careful purification and process optimization. Common downstream techniques, such as ammonium sulfate precipitation, ion exchange, and gel-filtration chromatography, enhance both enzyme purity and activity. Simultaneously, upstream adjustments of pH, temperature, salinity, carbon and nitrogen sources, and agitation can greatly boost yields. Moreover, methods, such as response surface methodology and bioreactor scale-up have proven effective in increasing enzyme production and stability, supporting the commercial development of microbial proteases [21].

3.4. Molecular Characterization

The isolate C2 showing high protease production was identified through 16S rRNA sequencing is found to be *F. phosphorivorans*. The 16S *rRNA* gene sequence was used to carry out BLAST with the "nr"

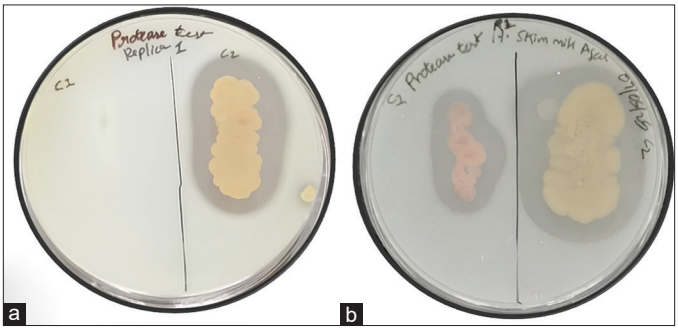

Figure 1: Primary protease screening of isolate C1 and C2 in skim milk (SM) agar plates (a) supplemented with 2.8% SM powder and (b) supplemented with 1% SM powder incubated at 37°C for 4 days.

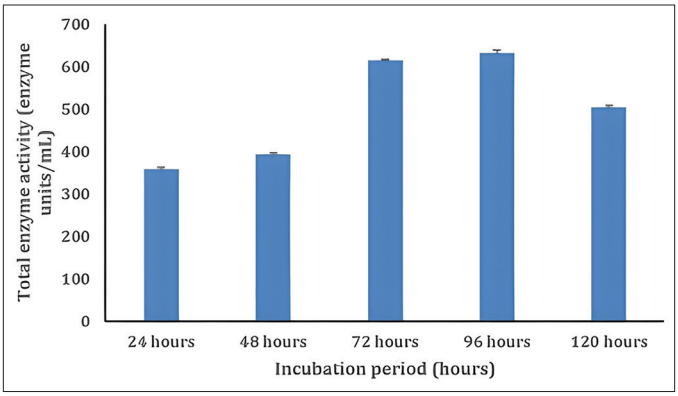

Figure 2: Total enzymes activity of the crude enzyme during various incubation periods (data expressed as mean ± standard deviation of 3 replicates [*n*=3]).

database of NCBI GenBank database and the accession number was obtained (Accession number: PX745081). Based on maximum identity score, first ten sequences were selected [Table 1] and aligned using multiple alignment software program Clustal W. Distance matrix and phylogenetic tree was constructed using MEGA 11. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura–Nei model. Bootstrap analysis was performed with 1000 replications, and bootstrap values above 50% are shown at branch nodes. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. The analysis included 11 nucleotide sequences with a total of 1519 positions in the final dataset [Figure 3]. The tree with the highest log likelihood (−2197.11) is shown. Initial

Table 1: List of ten sequences selected on the basis of maximum identity score with the isolate C2 for multiple sequence alignment using Clustal W.

Description	Max score	Total score	Query cover (%)	E (%)	Percentage identity	Accession
Fictibacillus phosphorivorans strain HTS	2787	2787	100	0.00	99.93	MG547923.1
Fictibacillus phosphorivorans strain pJ7	2780	2780	100	0.00	99.87	MW433865.1
Bacillus spp. 2049	2778	2778	100	0.00	99.74	JX566560.1
Bacterium B4 ZZ-2008 strain B4	2776	2776	100	0.00	99.80	FM180518.1
Bacillus nanhaiensis strain K-W9	2776	2776	99	0.00	99.87	JQ799063.1
Bacillus spp. STA	2776	2776	99	0.00	99.87	KC870063.1
Bacillus spp. LS23	2774	2774	100	0.00	99.80	FJ937886.1
Bacterium JL-74	2769	2769	99	0.00	99.80	AY745842.1
Bacillus arsenicus strain 58-06	2769	2769	100	0.00	99.74	EU620414.1
Bacillus nanhaiensis strain K-W28	2769	2769	99	0.00	99.80	JQ799081.1

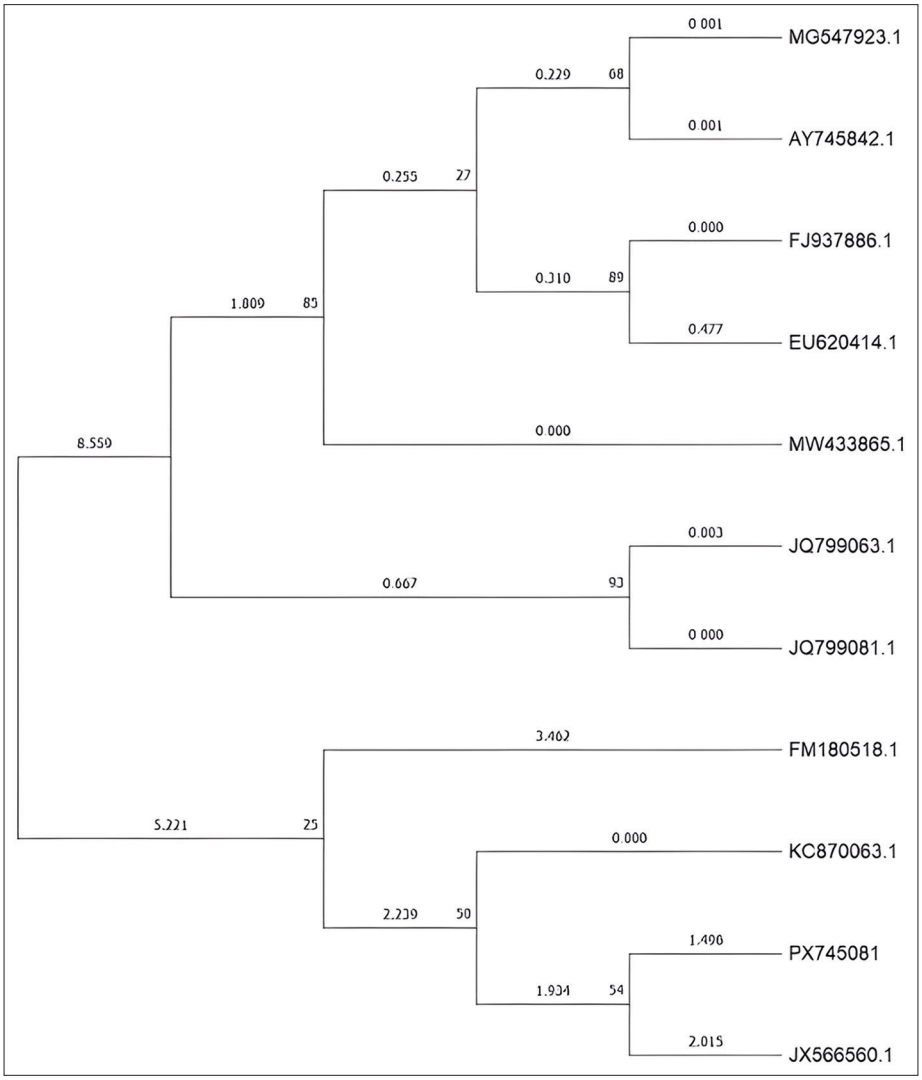

Figure 3: Evolutionary analysis of isolate C2 (PX745081) by maximum likelihood method.

tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value.

In this study *F. phosphorivorans*, isolated from mangrove soil, was reported to have high extracellular protease and keratinase activities – traits rarely reported in this genus. While previous research on *Fictibacillus* species mainly addressed phosphate solubilization, alkali tolerance, and plant growth promotion, there has been limited evidence of their significant proteolytic or keratinolytic abilities. Isolation of *F. phosphorivorans* in mangrove environments characterized by high organic matter, high salinity, and variable redox conditions suggests niche-specific adaptations that enhance enzyme production. This expands the known functional diversity of the *Fictibacillus* genus and highlights this strain as a promising source of industrially valuable proteases and keratinases [22].

3.5. Detergent Application by Blood Stain Removal Study

The most effective destaining results were obtained from the blood-stained fabric treated with a solution of detergent and crude enzyme combined. There was little stain removal in the control setting. In comparison with the control, the enzyme and detergent alone treatment settings demonstrated a partial elimination of the blood stain [Figure 4].

Similarly, a study conducted by Tian *et al*. showed that the stain removal efficiency was enhanced when crude protease was used as a detergent additive. When interacting with oxygen, hemoglobin present in the blood induces clot formation, which poses difficulty in removing the stain. When the protease breaks the peptide bonds, it is easy for the detergent to remove small amino acid chains [23]. The cleaning effects have increased significantly (35–40%) when alkaline protease was used as an additive in the detergent [24]. The protein stain cannot be effectively removed by adding surfactant without the enzymes. Therefore, the inclusion of enzymes in the formulation is essential for eliminating protein stains since they can break down the stain and facilitate its removal [25]. When mixed with detergents, protease creates amphipathic structures that facilitate the degradation of protein and fatty stains on fabric [26]. Microbial proteases used in laundry detergents to remove blood stains are degradable because they can naturally decompose in the environment without any hazardous residues, in contrast to conventional chemicals that can contaminate water and damage ecosystems [27].

3.6. Screening for Keratinolytic Activity by Feather Degradation

Poultry wastes, such as feathers, may be a great source of carbon, sulfur, and nitrogen that can be transformed into other beneficial products [28]. As a rich source of hydrolyzed proteins, chicken feathers can be utilized in the production of bioplastic films, biodegradable materials, high-nitrogen fertilizers, animal feed, and bioenergy [29]. After four days of incubation in the minimal media, substantial degradation of chicken feathers was observed. The majority of the feather substrate was hydrolyzed, resulting in a clear reduction in visible feather residues, demonstrating efficient enzymatic breakdown of feather keratin under the tested conditions. Approximately 86% of the feathers were completely degraded after 4 days of incubation [Figure 5]. In a study conducted by Riaz *et al*., 98% of feather degradation was observed after 7 days of incubation [30]. The primary byproducts of feather hydrolysis are found to be peptides and amino acids, which show properties of antioxidants that, either scavenge or defend against free radicals; hence, chicken feathers have a significant potential as a source of proteins and amino acids [31]. Keratin, a class of proteins with a high sulfur concentration, makes up 90% of chicken feathers, with bonds of disulfide between cysteine residues, as well as hydrophobic and ionic interactions, which give keratin its rigid structure [32].

Keratinases are a group of proteolytic enzymes that have a dual function as disulfide reductases and proteases, which cleave disulfide bonds between cysteine residues and peptide bonds between amino acids, aiding in the easy degradation of chicken feathers [33]. The amount of poultry waste, particularly feathers (which contain 90% keratin protein), has made them one of the main pollutants. Every year, the poultry industry produces approximately 8.5 million tons of feathers as a byproduct worldwide [34]. The most commonly used method, due to its simplicity, landfilling releases harmful gases (including nitrous oxide, hydrogen sulfide, and ammonia) from uncontrolled anaerobic

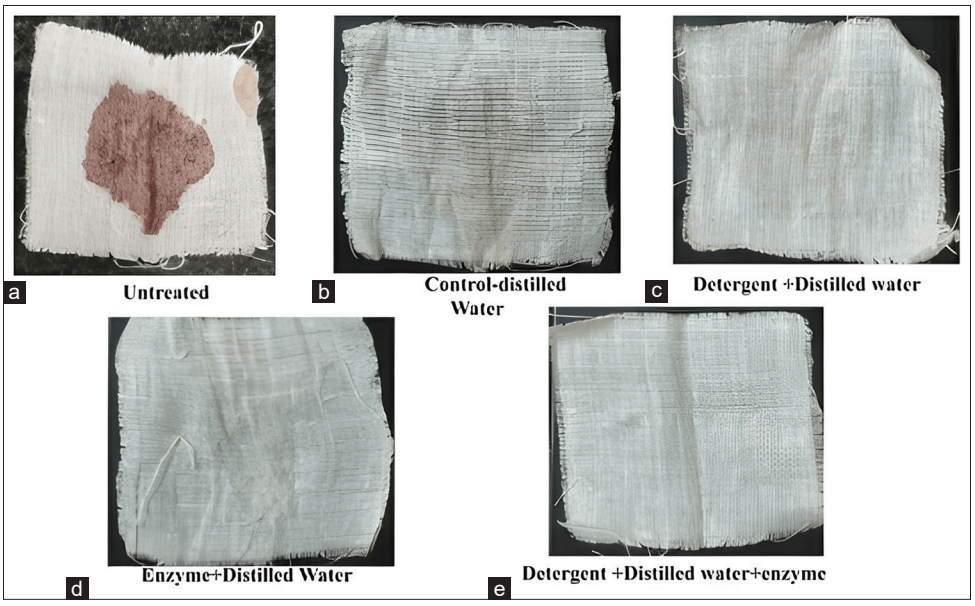

Figure 4: (a-e) Protease detergent application screening (immersed in the corresponding solutions for 30 min at 40°C).

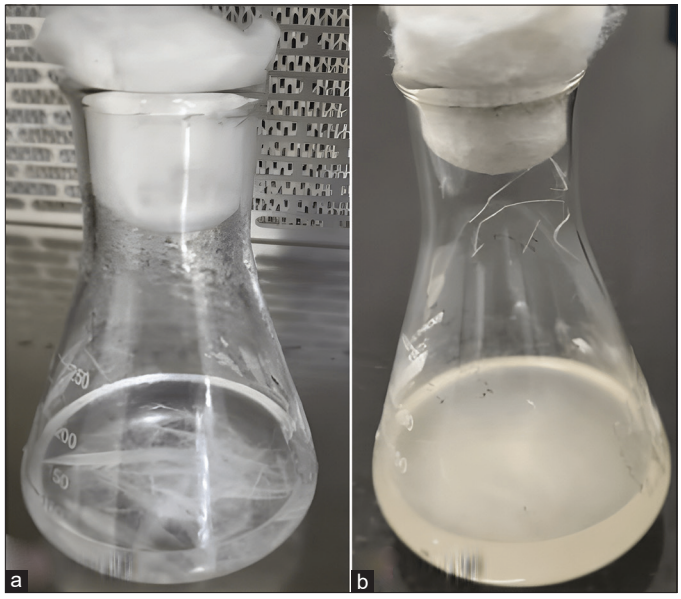

Figure 5: Feather degradation by isolate C2 (a) Before inoculation (b) after inoculation and incubation at 37°C with shaking (125 rpm) for 4 days showing 86% degradation.

digestion, pollutes groundwater, and results in soil infertility. They aggravate a number of diseases, including keratoconjunctivitis, Newcastle disease, poultry cholera, mycoplasmosis, air sacculitis, and chlorosis. Incineration is an option, although it has drawbacks, including issues with ash disposal and hazardous gas emissions. Hence, enzyme method can be a safe alternative as these feathers can be converted into industrially valuable products, hence avoiding the consequences posed by traditional methods [35].

4. CONCLUSION

The present study shows that mangrove soil from the Pichavaram region of Tamil Nadu is a valuable ecological spot for isolating bacteria that produce proteases. Among the screened isolates, *F. phosphorivorans* stood out as a promising candidate, showing the highest protease production of 633.14 U/mL after 96 h of incubation. The enzyme from this strain was effective in removing blood stains and displayed keratin-digesting activity, highlighting its potential applications in industries such as detergents, leather processing, and waste management. These results highlight the untapped potential of mangrove microbial communities. They also set the stage for further research to enhance enzyme production and explore other potential industrial applications. Using these local bacterial resources can help develop sustainable and cost-effective processes, emphasizing the need to conserve and study unique ecosystems, such as mangroves for future innovations in biotechnology.

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest related to this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

11. PUBLISHER'S NOTE

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12. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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